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**EP 0 359 831 A1**

54 **BIOSENSOR AND PROCESS FOR ITS PRODUCTION.**

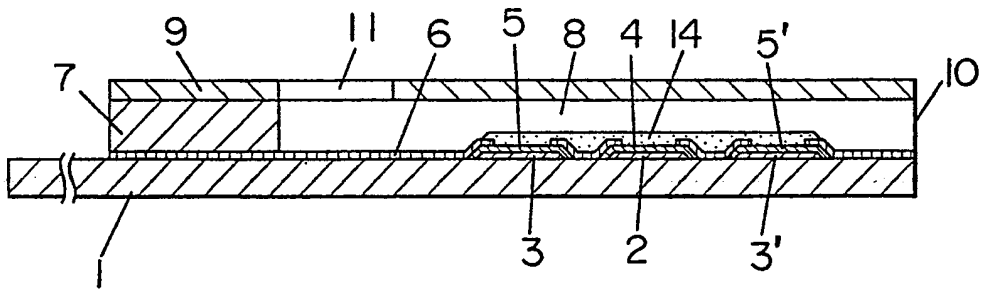
57 A biosensor of the invention comprises an insulating base board (1) having formed thereon, in sequence, leads (2, 3, 3'), an electrode system mainly made of carbon (4, 5, 5'), an insulating layer (6) and a reaction layer (14) composed of an enzyme and an electron acceptor, and being provided thereon with a space (8) defined by a spacer (7) and a cover (9). When a biological sample solution is

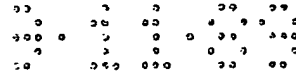
brought into contact with the inlet (10) of the biosensor having the above-described structure, the sample solution is introduced into its inside, while the air within the space (8) is rapidly discharged through the outlet (11) and, at the same time, the space (8) is filled with the sample solution up to the neighborhood of the outlet. Thus, measurement can be conducted inexpensively at a high speed with a high

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accuracy through simple procedures without residual bubbles.

FIG. 6





## SPECIFICATION

## 1 TITLE OF THE INVENTION

BIOSENSOR AND A PROCESS FOR PREPARATION  
THEREOF

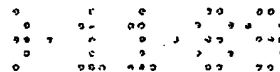
## FIELD OF THE INVENTION

- 5 The present invention relates to biosensors which can quantitatively determine a specific component in various sample solutions from the living body in a rapid and easy way with high accuracy and a process for preparation thereof.

## 10 BACKGROUND OF THE INVENTION

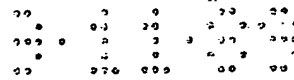
In recent years, various biosensors utilizing a specific catalytic action possessed by enzyme have been developed and in particular, it has been attempted to apply biosensors to the clinical field. In the present days when inspection items and sample numbers are increasing, biosensors which can provide rapid assay with good accuracy have been desired.

Taking a glucose sensor as an example, diabetes has markedly increased nowadays and for measurement and control of blood sugar level in blood, it takes a very long time, since blood is centrifuged and plasma is provided for the measurement as is conventionally done. Thus, a sensor which can make



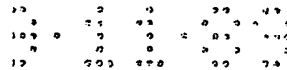
1 measurement with whole blood is required. As a handy  
type, there is a stick-like support having provided  
thereon a carrier containing an enzyme capable of  
reacting only with glucose and a dye which causes a  
5 change upon enzyme reaction or by the product of the  
enzyme reaction, like a test sheet used for inspection  
of urine. The stick takes the system that blood is  
dropped onto the carrier and after a definite period of  
time, a change of the dye is visually or optically  
10 determined. However, interference is serious because of  
colored matters in blood, resulting in poor accuracy.

Now, a multilayer type analysis carrier as  
shown in Fig. 1 is proposed (Japanese Utility Model  
Application Laid-Open No. 54-178495). The carrier has  
15 the construction comprising a transparent support 51  
having provided thereon, in order, a reagent layer 52, a  
spreading layer 53, a waterproofing layer 54 and a  
filtering layer 55. The measurement takes the following  
system: when a blood sample is dropped from the upside,  
20 solid components in blood such as red blood cells,  
platelets, etc. are removed by the filtering layer 55,  
the blood uniformly permeates into the spreading layer  
53 through a hole 56 in the waterproofing layer and a  
reaction proceeds in the reagent layer 52. After  
25 completion of the reaction, a light is irradiated from  
the arrow direction through the transparent support 51,  
whereby a substrate concentration is determined by  
colorimetry. The system takes a complicated



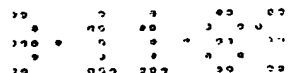
1 construction as compared to the conventional handy  
stick-like carrier but its accuracy has improved because  
blood cells are removed, etc. However, it takes a long  
time for the permeation of blood and the reaction so  
5 that the waterproofing layer 54 that prevents drying of  
the sample is required. In addition, incubation at a  
high temperature is required for accelerating the  
reaction. Thus, the system involves problems that  
apparatuses and carriers become complicated.

10 On the other hand, as the system for quantita-  
tive assay of a specific component in a sample such as  
blood, etc. from the living body with high accuracy  
without performing operations such as dilution, agita-  
tion, etc. of the sample solution, a biosensor as shown  
15 in Fig. 2 has been proposed (for example, Japanese  
Patent Application Laid-Open No. 59-166852). The  
biosensor comprises an insulating base plate 63 having  
embedded therein an electrode for measurement 64 and a  
counter electrode 65 made of platinum, etc., having  
20 leads 61 and 62, respectively, and the exposed areas of  
these electrodes are covered with a porous material 66  
having carried thereon an oxidoreductase and an electron  
acceptor. When a sample solution is dropped onto the  
porous material, the oxidoreductase and the electron  
25 acceptor are dissolved in the sample solution, whereby  
an enzyme reaction proceeds with a substrate in the  
sample solution and the electron acceptor is reduced.  
After completion of the reaction, the reduced electron



1 receptor is electrochemically oxidized and a substrate  
concentration in the sample is determined from a current  
level for the oxidation obtained in this case. In such a  
construction, however, the electrodes require operations  
5 such as washing, etc., while the porous material can be  
exchanged for every assay thereby to readily provide for  
measurement. On the other hand, if it is possible to  
dispose the apparatus including the electrode system for  
every measurement, operations for the measurement become  
10 extremely simple but from aspects of electrode materials  
such as platinum, etc., construction and the like, the  
apparatus is very expensive unavoidably. For the  
construction of platinum electrodes, the sputtering  
method or the deposition method can also be used but  
15 production costs increase.

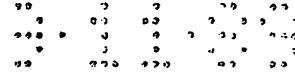
As a disposal system including the electrode  
system, a biosensor described in Japanese Patent  
Application Laid-Open No. 61-294351 has been proposed.  
As shown in Fig. 3, in this biosensor, the electrode  
20 systems 72 (72'), 73 (73') and 74 (74') composed of  
carbon, etc. are formed on an insulating base plate 71  
by means of screen printing, etc.; after providing an  
insulating layer 75 thereon, the electrode systems are  
covered with a porous material 77 having carried thereon  
25 an oxidoreductase and an electron acceptor and the whole  
is integrated with a holding frame 76 and a cover 78.  
When a sample solution is dropped onto the porous  
material, the oxidoreductase and the electron acceptor



1 are dissolved in the sample solution, whereby an enzyme  
reaction proceeds with a substrate in the sample  
solution and the electron acceptor is reduced. After  
completion of the reaction, the reduced electron  
5 acceptor is electrochemically oxidized and a substrate  
concentration in the sample is determined from a current  
level for the oxidation obtained in this case.

In the foregoing conventional construction,  
the base surface including the electrode system is not  
10 always uniformly wetted so that air bubbles remain  
between the porous material and the base plate, whereby  
a response current is affected or its reaction rate is  
reduced in some occasion. Further when an ambient  
humidity is low, moisture in a sample solution  
15 evaporates off during measurement so that a change in  
response is observed. Furthermore, when a substance  
that is readily adsorbed to electrodes or a substance  
such as ascorbic acid or the like that is easily  
oxidized is present in a sample solution, response of  
20 the sensor is affected by such a substance.

Therefore, as sensors for measuring a specific  
component in a vital sample solution such as blood or  
the like in a simple and rapid way with high accuracy,  
sensors which can provide measurement data merely by  
25 adding a trace amount of sample solution thereto without  
diluting or quantitatively determining the sample  
solution previously are desired. Furthermore,



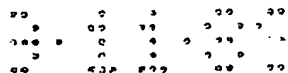
- 1 disposable type sensors which are free of operations  
such as washing, wiping, etc. are desired.

#### DISCLOSURE OF THE INVENTION

The biosensor of the present invention  
5 comprises an insulating base plate having provided  
thereon the electrode system comprised of at least an  
electrode for measurement and a counter electrode and on  
the electrode system a reaction layer containing an  
enzyme and an electron acceptor. Further by integrating  
10 with a cover, a space including the reaction layer is  
formed and an introducing port for introducing a sample  
solution into the space and a discharge port for  
discharging a gas in the space by inflow of the sample  
solution. A change of a substance in concentration  
15 caused by the reaction between the enzyme and the sample  
solution is detected by the electrode system thereby to  
determine a substrate concentration in the sample  
solution.

Furthermore, the electrode system may also be  
20 comprised of a plural set of electrode systems and  
reaction layers corresponding to the plural set of  
electrode systems are provided, whereby the independent  
sensor systems can be constructed. By doing so, two or  
more substrate concentrations can be concurrently  
25 determined. Furthermore, two pairs of electrode systems  
may also be used, wherein one electrode system is used  
for measurement and another electrode system is used for





1 blank. By determining a difference in response between  
the electrode systems, interference by ascorbic acid,  
etc. can be eliminated.

Further by integrating the base plate, the  
5 electrode system and the reaction layer with the cove, a  
space is formed and the surface of members constructing  
the space is rendered hydrophilic, whereby a definite  
quantity of sample solution can be readily introduced  
into the space described above through a simple  
10 operation merely by bringing a trace amount of sample  
solution into contact with the introducing port of the  
sensor.

The introduced sample solution dissolves the  
electron acceptor, enzyme and hydrophilic high molecular  
15 substance in the reaction layer, where an enzyme  
reaction proceeds while converting the sample solution  
into a viscous liquid. By the enzyme reaction, a  
reduced electron acceptor is produced in response to the  
substrate concentration in the sample solution. Then,  
20 the reduced electron acceptor is electrochemically  
oxidized on the electrode for measurement to determine  
the substrate concentration from a current level for the  
oxidation.

In the biosensor of the present invention, a  
25 hydrophilic high molecular substance is used so that  
influence of solid components such as red blood cells  
and the like in a blood sample on the electrodes can be  
prevented and at the same time, by providing the space,



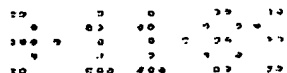
1 fluidization of the sample solution on the electrodes  
can be effectively prevented. Thus, an influence by  
vibration during measurement can be prevented.

On the other hand, the reaction layer in the  
5 biosensor of the present invention is comprised of an  
enzyme layer and an electron acceptor layer provided  
thereon. Further by providing a hydrophilic high  
molecular substance layer between these layers, the  
enzyme and the electron acceptor are separated from each  
10 other to provide a biosensor having excellent  
preservation property.

According to the present invention, a  
disposable biosensor including the electrode system can  
be constructed so that a substrate concentration in the  
15 sample, for example, a glucose concentration in blood  
can be determined rapidly in a simple operation with  
high accuracy, merely by adding an extremely trace  
amount of sample solution, without diluting or  
quantitatively determining the sample solution in  
20 advance.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an illustrative drawing showing an  
example of conventional glucose sensors. Figs. 2 and 3  
illustratively show glucose sensors using conventional  
25 enzyme electrodes. Fig. 4 shows a perspective view of a  
disassembled biosensor which is one embodiment of the  
present invention. Fig. 5 shows an external view of the



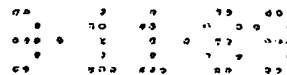
1 biosensor. Fig. 6 illustratively shows a cross-sectional view of the biosensor. Fig. 7 shows a response characteristic of the biosensor. Fig. 8 shows a perspective view of a disassembled biosensor which is  
5 another embodiment of the present invention. Fig. 9 shows an external view of the biosensor. Figs. 10, 12 and 13 show perspective views of disassembled biosensors which are other embodiments of the present invention. Fig. 11 shows a perspective view of a disassembled  
10 biosensor which is another embodiment of the prior art. Fig. 14 shows a relationship between ascorbic acid concentration and response characteristic. Fig. 15 shows a relationship between the particle diameter of potassium ferricyanide and the time when the reaction is  
15 completed.

#### BEST MODES FOR PRACTICING THE INVENTION

##### Example 1

In the following explanatory drawings in the examples, the same numbering is used for common elements  
20 and their explanation is in part omitted.

As one embodiment of the biosensor, a glucose sensor is explained. Fig. 4 shows a perspective view of a disassembled glucose sensor prepared as one embodiment of the biosensor in accordance with the present  
25 invention. Fig. 5 shows an external view of the biosensor. Fig. 6 illustratively shows a cross-sectional view of the biosensor obtained when the



1 biosensor shown in Fig. 5 is cut at the center into the  
length direction. In Fig. 4, a reaction layer 14 shown  
in Fig. 6 is not shown.

Hereafter a process for preparing the sensor  
5 is described. Silver paste is printed on an insulating  
base plate 1 composed of polyethylene terephthalate by  
means of screen printing to form leads 2, 3 (3'). Next,  
conductive carbon paste containing a resin binder is  
printed thereon. By drying with heating, the electrode  
10 system comprised of an electrode for measurement 4 and a  
counter electrode 5 (5') is formed. Furthermore,  
insulating paste is printed so as to partly cover the  
electrode system to make the exposed area of the  
electrodes definite and cover unnecessary part of the  
15 leads. By a heat treatment, an insulating layer 6 is  
formed.

Next, the exposed area of the electrode system  
4, 5 (5') is polished and then heat-treated at 100°C for  
4 hours in the air. After the electrode portion is thus  
20 constructed, 0.5% aqueous solution of carboxymethyl  
cellulose (hereafter simply referred to as CMC) as the  
hydrophilic high molecular substance is spread onto the  
electrodes and dried to form a CMC layer. A solution of  
glucose oxidase (GOD) as the enzyme in phosphate buffer  
25 solution is spread thereon and dried to form a reaction  
layer 14 comprised of CMC-GOD layer. In this case, CMC  
and GOD form a thin layer having a thickness of several  
microns in a partly mixed state.



1           Next, three members of this base plate 1, a  
spacer 7 comprised of a resin plate and a cover 9 are  
adhered to each other in such a positional relationship  
between the respective members shown by broken lines as  
5 shown in Fig. 4 to integrate the sensor as shown in the  
external view in Fig. 5. Herein, the spacer has a  
thickness of approximately 300  $\mu\text{m}$  and takes a shape that  
the central part is cut off into a U-(dai) shape having  
a width of 2 (dai) and a length of 7 mm. The end of the  
10 cut portion becomes an introducing port 10 of a sample  
solution and the central part forms a space 8, when  
integrated. Furthermore, the cover 9 possess a hole  
having a diameter of 2 (dai) and forms a discharge port  
11 when integrated.

15           When the introducing port at the tip of the  
glucose sensor constructed as described above is brought  
into contact with a glucose standard solution (200  
mg/d $\ell$ ) which is a sample solution, the sample solution  
is introduced into the inside through the introducing  
20 port 10. In this case, the air in the space 8 is  
rapidly discharged through the discharge port 11 and at  
the same time, the space is filled up with the sample  
solution up to near the discharge port. As such, the  
sample solution rapidly spreads onto the electrode  
25 surface to fill up the space so that any remaining air  
bubbles are not noted.

This is believed to be because the sample  
solution would flow into one direction by providing the

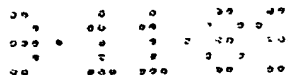


1 introducing port and the discharge port and due to the  
hydrophilic high molecular substance layer previously  
formed on the electrodes, wetting on the electrode  
surface would be improved so that the gas is smoothly  
5 exchanged with the liquid.

Further by previously treating the surfaces of  
the members constructing the space such as the cover,  
spacer, etc., with a surface active agent to render them  
hydrophilic, the sample solution can be introduced more  
10 smoothly.

On the other hand, the added sample solution  
dissolves CMC therein to render the liquid viscous.  
Glucose in the sample solution reacts with the enzyme by  
the action of glucose oxidase carried on the electrodes  
15 to produce hydrogen peroxide. Therefore, by applying a  
voltage of 1 V between the electrodes one minute after  
introduction of the sample solution, the electrode for  
measurement is polarized into the anode direction. By  
applying a voltage to the anode direction described  
20 above, an oxidizing current for the produced hydrogen  
peroxide is obtained. This current level corresponds to  
the concentration of glucose which is a substrate.

As an example of the response characteristic  
of the sensor constructed as described above, the  
25 relationship between a current level 5 seconds after  
application of voltage and a glucose concentration is  
shown in Fig. 7, A, indicating that a good response  
characteristic was obtained.



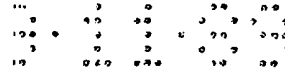
1 Further with respect to 30 glucose sensors  
having the same specification as described above, the  
same sample solution was tested with the sensors. A  
coefficient of variation was as good as about 5%.

5 Furthermore, influence by evaporation was  
examined under humidity conditions of 30% and 80%, using  
10 sensors each, and compared with the glucose sensor  
shown in Fig. 3 which was prepared in a conventional  
construction by way of trial. In the glucose sensors  
10 according to the present invention, the influence was  
negligible even after allowing to stand for 5 minutes.  
However, in the sensors having a conventional  
construction, the evaporation was higher by about 3%  
under the lower humidity condition 2 minutes after.

15 As described above, the glucose sensor of the  
present invention having the construction described  
above can rapidly absorb a trace amount of the sample  
solution into the reaction layer zone and can determine  
a glucose concentration in a simple and rapid way with  
20 high accuracy, without being affected by ambient  
humidity, etc.

#### Example 2

The procedure was quite the same as in Example  
1 until the CMC-GOD layer was formed. Thereafter, a  
25 reaction layer composed of a CMC-GOD-electron acceptor  
layer was further formed thereon by the following  
method.



1                    Surface active agent, lecithin (phosphatidyl  
choline), was dissolved in toluene to prepare 1 wt%  
solution and microcrystals (a mean particle diameter of  
less than 10  $\mu\text{m}$ ) of potassium ferricyanide as an  
5 electron acceptor was dispersed in the solution. Then  
toluene was evaporated off to form the reaction layer  
composed of CMC-GOD-electron acceptor layer on the  
electrode system followed by integrating with a spacer  
and a cover as in Example 1. Thus, a glucose sensor  
10 having the construction shown in Fig. 5 was obtained.

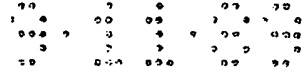
Next, a glucose standard solution was  
introduced through the tip portion of the sensor as  
described above. By applying a voltage of 600 mv  
between the electrodes about one minute after, potassium  
15 ferricyanide produced by the enzyme reaction was  
oxidized on the electrode for measurement. A current  
level in this case was measured 5 seconds after  
application of the voltage. As the result, an extremely  
good linear relationship was obtained between the  
20 glucose concentration and the response current level, as  
shown in Fig. 7, B. Further using 30 sensors, a  
variation coefficient was examined. The variation  
coefficient was 2.6% with respect to the glucose  
standard solution and 3% with respect to the whole blood  
25 sample, which were extremely good. Further with respect  
to influence of evaporation, a test was carried out in a  
manner similar to Example 1 and similar effects were  
obtained, as described above.





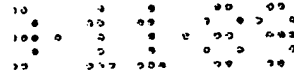
1            In the above example, when the concentration  
of lecithin was greater than 0.01 wt%, potassium  
ferricyanide was efficiently dispersed in toluene so  
that dropping became easy to form a thin potassium  
5 ferricyanide-lecithin layer. Where no lecithin is  
present, defects that the potassium ferricyanide layer  
was non-uniformly formed or the base plate was peeled  
off when bent were noted. However, by incorporating  
lecithin, the potassium ferricyanide layer which was  
10 uniform and peeled off only with difficulty could  
readily be formed. As the concentration of lecithin  
increased, the potassium ferricyanide layer was peeled  
off more difficultly but a dissolution rate of potassium  
ferricyanide also decreased. Therefore, a suitable  
15 concentration is believed to be 0.01 to 3 wt%.

Polyethylene glycol alkyl phenyl ether (trademark:  
Triton X) was used instead of lecithin. In order to  
disperse fine particles of potassium ferricyanide in  
toluene, more than 0.1% was necessary but a good  
20 potassium ferricyanide layer could be formed as in the  
case of using lecithin. As the surface active agent,  
there are oleic acid, polyoxyethylene glycerine fatty  
acid ester, cyclodextrin, etc., in addition to the  
example described above. Surface active agents are not  
25 particularly limited so long as they can disperse the  
electron acceptor in an organic solvent and do not  
affect the enzyme activity. As the organic solvent for  
mixing the electron acceptor therewith, solvents such as



1 toluene, petroleum ether, etc. may be used as long as  
they have a minimized influence on GOD activity and the  
printed electrodes.

With respect to the particle diameter of  
5 microcrystalline potassium ferricyanide used above,  
commercially available crystals of potassium  
ferricyanide were ground into powders and crystals of a  
definite particle diameter were collected by sieving to  
form a potassium ferricyanide layer. With respect to  
10 the same glucose sensors as described above prepared  
from crystals of various particle diameters, their  
responses were compared with each other. Fig. 15 shows  
a mesh size of sieve on the abscissa and on the  
ordinate, a time for completing the reaction to 400  
15 mg/dl of glucose. Numerals with parentheses indicate a  
size ( $\mu\text{m}$ ) of the mesh hole. As shown in Fig. 15,  
crystals having a smaller particle size were dissolved  
more quickly and the time required for completing the  
reaction was shorter. In the sensor prepared with  
20 potassium ferricyanide (particle size of 100  $\mu\text{m}$  or  
less) passed through 145 mesh (Japanese Industrial  
Standard), the reaction was completed within 2 minutes.  
In addition, when the potassium ferricyanide layer  
was prepared, crystals having a smaller particle  
25 diameter could form a uniform layer and provided less  
unevenness in response. Microcrystals of potassium  
ferricyanide could be formed by grinding into powders  
but recrystallization of an aqueous potassium

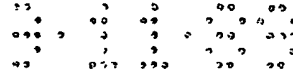


1 ferricyanide solution from ethanol could easily prepare  
crystals having a particle diameter of not greater than  
10  $\mu\text{m}$ . When the potassium ferricyanide layer was formed  
from such crystals, the layer became dense and the time  
5 for completing the reaction could be shortened.

When potassium ferricyanide finely divided  
into a particle diameter of less than 100  $\mu\text{m}$  was mixed  
with toluene and the mixture was dropped, toluene was  
rapidly evaporated and the potassium ferricyanide layer  
10 could be formed in a microcrystalline state so that a  
dissolution rate was rapid and rapid measurement was  
attained. Further by using the organic solvent, the  
potassium ferricyanide layer could be formed separately  
on the CMC-GOD layer, whereby preservation property  
15 could be improved.

In providing the introducing port and the  
discharge port in the example described above, in  
addition to the arrangement shown in Figs. 4 through 6,  
holes may be provided in the cover 9 and the base plate  
20 1, respectively, and used as the introducing port 10 and  
the discharge port 11 as shown in the perspective view  
of the disassembled sensor in Fig. 8 and in the external  
view in Fig. 9.

Furthermore, as shown in the perspective view  
25 of the disassembled sensor in Fig. 10, the spacer may be  
divided into two parts of 7 and 7' and the parts may be  
used as the spacer 8, and the introducing port 10 and  
the discharge port 11.

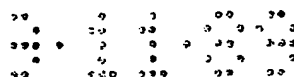


1 Furthermore, the shape of the tip portion of  
the biosensor having the structure shown in Fig. 5 was  
rounded as shown in the external view shown in Fig. 11.  
By doing so, a portion brought into contact with a  
5 sample solution was limited almost to the introducing  
port 10 when the sample solution was introduced. Thus,  
the sample solution did not go around the periphery of  
the introducing port but could be smoothly introduced  
even in a small quantity.

10 Example 3

The electrode portion shown in Fig. 12 was  
constructed in a manner similar to Example 1. That is,  
silver leads 3, 21, 22 and 23, counter electrode 3  
corresponding thereto and three electrodes for  
15 measurement 41, 42 and 43 were formed on a base plate to  
construct three pairs of electrode systems possessing  
the counter electrode in common. Next, a reaction layer  
composed of CMC-GOD-electron acceptor layer was formed  
around each of the electrodes for measurement in a  
20 manner similar to Example 2.

Then, a spacer and a cover were adhered to  
integrate them as in Example 1. Herein, the spacer is  
partly cut off into a "comb-like" shape and the cut  
portion forms a space 8. The space is divided into the  
25 respective electrode systems but is continuous as a  
whole to form a common space. Furthermore, the end  
becomes the introducing port 10.



1           In response to the space dividedly constructed  
on these respective electrode systems, three holes are  
formed in the cover 9 to provide discharge ports 11, 12  
and 13. When the introducing port 10 is brought into  
5 contact with a sample solution, the sample solution is  
rapidly introduced into the space, while discharging the  
air in the space through the three discharge ports.

A response to the glucose standard solution  
was measured with respect to the three electrode systems  
10 in a manner similar to Example 2 and its mean value was  
determined to make it a response level for one sensor.  
The test was performed with 30 glucose sensors having  
the same specification. The variation coefficient was  
as good as 2%.

15           In the disposable type sensor, it is important  
for improved reliability to minimize factors for error  
including a difference in electrode area of each sensor.  
The difference between the sensors forms a cause for  
causing a difference in error of measurement data on the  
20 same sample solution. However, as described above,  
measurement with higher accuracy can be rapidly achieved  
by providing a plurality of electrode systems for the  
same sensor and obtaining a mean value of the response  
levels.

25           Fig. 12 shows the embodiment having 3 pairs of  
electrodes but the present invention is not limited  
thereto and can further provide a large number of  
electrode systems. Furthermore, the shape of the space

1 is not limited to those shown in the figures, likewise  
the shape or arrangement of the electrode systems.

#### Example 4

Fig. 13 shows a variation of the construction  
5 having a plurality of the electrode systems. In a  
manner similar to Example 1 already described, silver  
leads 21, 22 and 3 and two pairs of the electrode  
systems comprised of electrodes for measurement 41 and  
42 corresponding thereto and a counter electrode 5 in  
10 common were constructed on the base plate. Next, as in  
Example 2, after a reaction layer composed of CMC-GOD-  
potassium ferricyanide layer was formed on each of the  
electrode systems, a spacer 7 and a cover 9 were adhered  
thereto. Herein, the spacer has two holes which  
15 independently form spaces 81 and 82 and, discharge ports  
11 and 12 corresponding to the holes and introducing  
port 10 used in common are provided in the cover.

With respect to the glucose sensor having the  
construction described above, as shown in Fig. 3, an  
20 average was taken from response values of two pairs of  
the electrode systems to the glucose standard solution  
and made a measurement value for one sensor. When 30  
sensors having the same specification were tested, the  
variation coefficient was as good as 2.2%.

25 As such, in the sensor shown in Fig. 13,  
measurement can be made with higher accuracy. In  
addition, since the sensor possesses the independent

1 spaces, different sample solutions can be measured with  
one sensor or the sensor can be provided for measurement  
under different conditions in time, temperature, etc.

In the example described above, one hole was  
5 provided in the cover to make it a common introducing  
port but the present invention is not limited to this  
embodiment. It is important to construct the  
measurement system comprising a plural pairs of the  
electrode systems and a plurality of independent spaces.  
10 Therefore, two holes may also be provided and  
introducing ports to the respective spaces may be  
constructed.

#### Example 5

Using the electrode part, spacer and cover  
15 having constructions shown in Fig. 13, a glucose sensor  
described below was constructed. Firstly, a reaction  
layer composed of CMC-GOD was formed on an electrode for  
measurement 41 in a manner similar to Example 1. On the  
other hand, CMC layer alone was formed on an electrode  
20 for measurement 42.

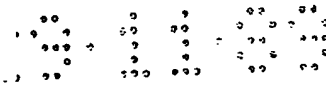
With respect to the glucose sensor having two  
pairs of the electrode systems obtained as described  
above, a glucose standard solution (200 mg/dℓ)  
containing ascorbic acid having various concentrations  
25 was dropped onto introducing port 10 and introduced onto  
each of the electrode systems. Next, as in Example 1, a  
voltage of 1 V was applied about 1 minute after the

1 dropping and a current level was measured 5 seconds  
after. The results are shown in Fig. 14. The output of  
the electrode system of CMC-GOD layer is shown by A and  
the output (blank output) of the electrode system of CMC  
5 layer alone is shown by B. As is evident from the  
drawing, the output of A increases as the concentration  
of ascorbic acid increases and on the other hand, a  
similar increase is noted with the output of B. This  
indicates that the sensitivities of the respective  
10 electrode systems to ascorbic acid are almost equal to  
each other. When a difference in output between the  
both electrode systems (A - B) is detected therefrom, a  
current level based on glucose can be obtained. That  
is, by using two pairs of the electrode systems, an  
15 error due to substances sensitive to electrode can be  
greatly reduced. Such an effect was also noted with  
uric acid, etc., in addition to ascorbic acid.

As such, by constructing the sensor by  
providing two pairs of the electrode systems and forming  
20 a hydrophilic high molecular substance-enzyme layer on  
one electrode system and a hydrophilic high molecular  
substance layer alone on another electrode system, a  
substrate concentration in the sample solution  
containing interferants can be measured with good  
25 accuracy.

In the above, after the CMC-GOD layer is  
formed on both electrode systems, local heating by laser  
or irradiation with ultraviolet rays, etc. may also be



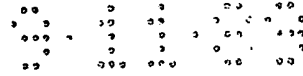


1 applied only to either electrode system, whereby GOD is  
inactivated to prepare the electrode system for blank  
outputting. By doing so, the constructions are  
identical in the two electrode systems except for enzyme  
5 activity so that output currents due to interferants in  
the two electrode systems can be conformable much better  
with each other, resulting in an improved accuracy in  
detection with the sensor.

In the foregoing embodiment, the electrode  
10 system wherein the electrode portion comprises two  
electrodes of the electrode for measurement and the  
counter electrode has been described. By constructing  
the electrode system by three electrodes further  
involving silver/silver chloride, the accuracy can  
15 further be improved. One embodiment for constructing  
the electrode system comprises printing 3 silver leads  
onto a base plate, then printing a carbon paste only on  
the tip portions of two leads to coat an insulating  
layer, treating the surface of the tip portion of the  
20 remaining lead in which silver is exposed to form silver  
chloride into a silver/silver chloride electrode. Thus,  
the electrode system could be constructed in such a  
manner.

#### Example 6

25 In Example 5, a reaction layer composed of  
CMC-GOD-potassium ferricyanide layer and a layer  
composed of CMC-potassium ferricyanide were formed on



1 the electrode for measurement 41 and the electrode for  
measurement 42, respectively, in a manner similar to  
Example 2. Measurement was performed in a manner  
similar to Example 5 except that the voltage applied was  
5 0.6 V. Influence of ascorbic acid could be removed as  
described above.

#### Example 7

In Example 5, a reaction layer composed of  
CMC-GOD-potassium ferricyanide layer was formed on the  
10 electrode for measurement 41 in a manner similar to  
Example 2. Further on the electrode for measurement 42,  
the CMC layer was formed and an enzyme layer composed of  
glucose oxidase, mutarotase and B-glucosidase was then  
formed thereon and an electron acceptor layer composed  
15 of potassium ferricyanide was further formed to make a  
reaction layer. Measurement was performed by applying a  
voltage of 0.6 V and using as a sample solution an  
aqueous solution containing glucose and sucrose. With  
the electrode for measurement 41, a current in response  
20 to glucose concentration was obtained and a current in  
response to the total concentration of glucose and  
sucrose was obtained with the electrode for measurement  
42. From a difference between these current levels, the  
sucrose concentration could be determined. As such, two  
25 substrate concentrations can be measured.

## 1 Example 8

After the electrode portion was prepared in a manner similar to Example 1, 0.5% aqueous solution of carboxymethyl cellulose (hereafter simply referred to as  
5 CMC) as the hydrophilic high molecular substance was spread onto the electrodes and dried to form a CMC layer. Next, a solution of glucose oxidase in water was spread thereon and dried to form a CMC-GOD layer. In this case, CMC and GOD formed a thin layer having a  
10 thickness of several microns in a partly mixed state. Furthermore, 0.5% ethanolic solution of polyvinylpyrrolidone (hereafter simply referred to as PVP) was spread so as to fully cover the first layer composed of this CMC-GOD layer and dried to form a  
15 second layer composed of PVP layer. A mixture of microcrystalline potassium ferricyanide as the electron acceptor and 1% solution of a surface active agent, lecithin, in toluene was dropped and spread onto the PVP layer and dried to form a third layer composed of  
20 potassium ferricyanide-lecithin layer followed by integrating with a spacer and a cover as in Example 1.

By using toluene in which PVP is sparingly soluble as a solvent in the case of forming the potassium ferricyanide-lecithin layer, it is possible to  
25 uniformly spread the potassium ferricyanide-lecithin solution onto the PVP layer. As the result, the uniform potassium ferricyanide-lecithin layer can be obtained.

As such, the use of a solvent in which the

1 hydrophilic high molecular substance constructing the  
second layer as the solvent for spreading the electron  
acceptor and the surface active agent could form the  
extremely uniform electron acceptor layer (third layer).

5 A glucose standard solution was introduced  
into the glucose sensor constructed as above through the  
introducing port. By applying a voltage of +0.6 V to  
the electrode for measurement in the anode direction one  
minute after, a response current was measured 5 seconds.

10 A good linear relationship was obtained up to the  
concentration as high as more than 900 mg/dℓ. Onto the  
glucose sensor described above, 5 μℓ of blood sample was  
dropped and a response current was measured one minute  
after. A response with very good reproducibility was  
15 obtained.

With respect to two sensors, i.e., the glucose  
sensor prepared by the process described above and the  
same glucose sensor except that the second layer  
composed of hydrophilic high molecular substance was not  
20 provided, preservation test was performed at 35°C for 30  
days in a dried state. Using a glucose standard  
solution (90 mg/dℓ) as a sample solution, sensor  
response was compared 30 days after. The sensor in  
which the second layer composed of the hydrophilic high  
25 molecular substance was not formed showed CV value of  
5.3 but the sensor in which the second layer composed of  
PVP showed CV value as extremely good as 2.5.

## 1 Example 9

The CMC-GOD layer and the PVP layer were prepared in a manner similar to Example 8. A mixture of microcrystalline potassium ferricyanide as the electron  
5 acceptor and 0.5% solution of a surface active agent, lecithin, in ethanol was dropped and spread onto the PVP layer and dried to form a potassium ferricyanide-  
lecithin layer. By using ethanol in which PVP is readily soluble as a solvent in the case of forming the  
10 potassium ferricyanide-lecithin layer, it is possible to concentratively develop at one point on the PVP layer. That is, it was possible to concentrate on the electrode for measurement of the sensor to construct the uniform  
potassium ferricyanide-lecithin layer so that the sensor  
15 in which a stable response was obtained merely by spreading the minimum quantity required could be prepared.

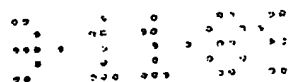
Response characteristics to the glucose standard solution of the glucose sensor constructed as  
20 above were determined in a manner similar to Example 8. A good linear relationship was obtained up to the concentration as high as more than 900 mg/dl. In addition, a response with very good reproducibility was obtained also in the case of using blood as a sample  
25 solution. Furthermore, with respect to the glucose sensor in which the second layer composed of hydrophilic high molecular substance was provided and the same glucose sensor except that the second layer composed of

1 hydrophilic high molecular substance was not provided,  
preservation test was performed at 35°C for 30 days in a  
dried state in a manner similar to Example 8. The  
sensor in which the second layer composed of PVP showed  
5 extremely good CV value, in the response measured 30  
days after.

In the prior art construction, the GOD-CMC  
layer was already in contact with the potassium  
ferricyanide-lecithin layer at the time when the sensor  
10 was prepared so that it was difficult to improve  
preservation efficiency. The hydrophilic high molecular  
substance layer composed of PVP which was used in  
Example 8 described above and present Example 9 takes a  
role to completely separate the GOD-CMC layer from the  
15 potassium ferricyanide-lecithin layer in a dried state.

In addition, the hydrophilic high molecular  
substance layer is extremely effective to ensure a  
stable sensor response, also in the case that substances  
readily adsorbed to the electrode or electrode-active  
20 substances are present in a sample solution. Even in  
the case that a glucose concentration was determined by  
the glucose sensor described above using blood as a  
sample solution, a stable sensor response was obtained  
irrespective of viscosity or the like of the sample  
25 solution.

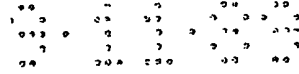
In Examples 1 through 9 described above, one  
embodiment of preferred positional relationship between  
the introducing port and the discharge port connected



1 with the space is illustratively shown in the drawings.  
The discharge port may also be used as the introducing  
port and the introducing port may be used as the  
discharge port. Furthermore, the spacers and the covers  
5 shown in the respective drawings are composed of  
independent materials but are not limited thereto. It  
is advantageous in mass production to form an integrated  
cover member with a shape of cover by means of molding  
or the like.

10 Further by rendering the surface of materials  
constructing the space hydrophilic, a sample solution  
was introduced more smoothly through the introducing  
port. The effect was obtained either by using  
hydrophilic materials or by previously treating the  
15 surface materials constructing the space with a surface  
active agent to render them hydrophilic.

On the other hand, an interval between the  
base plate and the cover can be controlled by varying  
the thickness of spacer or the size of a portion  
20 corresponding thereto. However, when the interval is  
too large, a quantity of sample solution required for  
filling up the space becomes large and it is also  
disadvantageous to introduce the sample solution by  
capillary phenomenon. When the interval is too small, a  
25 resistance between the electrodes increases or a current  
distribution is distorted. From the foregoing, the  
interval is preferably in a range of 0.05 to 1.0



1 (dai), more preferably 0.1 to 0.5 (dai).

In the examples, CMC was used as the hydrophilic high molecular substance. As has already been stated, its role is to prevent influence of adsorbable substances contained in a sample solution adsorbed to the electrode on response, in addition to the smooth introduction of the sample solution. Furthermore, its role also lies in separating the enzyme layer from the electron acceptor layer by the hydrophilic high molecular substance layer thereby to further improve preservation property. As the hydrophilic high molecular substance, gelatin, methyl cellulose and the like can be used, in addition to CMC, and hydrophilic high molecular substances of starch, carboxymethyl cellulose, gelatin, acrylate, vinyl alcohol, vinylpyrrolidone and maleic anhydride are preferred. These water-absorbing or water-soluble hydrophilic high molecular substances are dissolved in a suitable concentration and the resulting solution is coated and dried to form a hydrophilic high molecular substance layer having a necessary layer thickness.

In the foregoing examples, the electrode system in which the electrode portion is comprised of two electrodes of an electrode for measurement and a counter electrode has been stated. However, by constructing the electrode system with three electrodes further including a reference electrode, the accuracy can be further improved. As materials for the



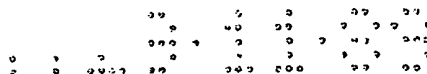
1 electrode, carbon illustrated in the examples is suitable as a stable and inexpensive material but the materials are not limited thereto. Other noble metal materials and oxidized materials may also be used.

5 Further as the electron acceptor, though potassium ferricyanide used in the examples is excellent in stability and reaction rate, redox compounds such as quinone compounds or ferrocene compounds, etc. can also be used, in addition to potassium ferricyanide.

10 Furthermore, the oxidoreductase is not limited to glucose oxidase shown in the foregoing examples but various enzymes such as alcohol oxidase, cholesterol oxidase, etc. can be used.

#### INDUSTRIAL APPLICABILITY

15 The biosensor of the present invention can rapidly determine a specific component in various sample solutions in an accurate and simple way. Therefore, its utilization value is extremely high in clinical inspection.



## CLAIMS

1. A biosensor for determining a substrate concentration in a sample solution comprising a base plate having an electrode system and a reaction layer having formed thereon a space including said reaction layer, said space being provided with an introducing port for introducing said sample solution into said space and a discharge port for discharging the gas in said space by inflow of said sample solution, said electrode system being equipped with at least an electrode for measurement and a counter electrode, at least an enzyme being carried on said reaction layer, a change in concentration of a substance in the reaction between said enzyme and said sample solution being detected with said electrode system to determine a substrate concentration in said sample solution.
2. A biosensor as claimed in claim 1, wherein said electrode system comprises a plural set of electrode systems and reaction layers corresponding to said plural set of electrode systems and a common space are provided.
3. A biosensor as claimed in claim 1, wherein said electrode system comprises a plural set of electrode systems and, reaction layers and spaces corresponding to said plural set of electrode systems are provided.
4. A biosensor as claimed in claim 1, wherein an electrode system comprising at least an electrode for

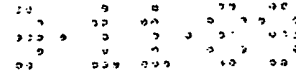
measurement and a counter electrode is formed on an insulating base plate, a reaction layer is formed on the surface of said electrode system and said reaction layer comprises an enzyme layer composed of an oxidoreductase and a hydrophilic high molecular substance having formed thereon an electron acceptor layer.

5. A biosensor as claimed in claim 1, wherein an electrode system comprising at least an electrode for measurement and a counter electrode is formed on an insulating base plate, a reaction layer is formed on the surface of said electrode system and said reaction layer comprises an enzyme layer composed of an oxidoreductase and a hydrophilic high molecular substance having formed thereon an electron acceptor layer containing a surface active agent.

6. A biosensor as claimed in claim 4 or 5, wherein said electron acceptor layer comprises fine particles of an electron acceptor having a particle size of not greater than 100  $\mu\text{m}$ .

7. A biosensor as claimed in claim 4 or 5, wherein said hydrophilic high molecular substance is any member selected from hydrophilic high molecular substances of starch, carboxymethyl cellulose, gelatin, acrylate, vinyl alcohol, vinylpyrrolidone and maleic anhydride or a mixture thereof.

8. A biosensor as claimed in claim 1, wherein the surface of a material constructing said space is



hydrophilic.

9. A biosensor as claimed in claim 2 or 3, wherein said electrode system comprises two sets of electrode systems comprising at least an electrode for measurement and a counter electrode mainly composed of carbon, a reaction layer composed of a hydrophilic high molecular substance and an oxidoreductase being provided on one electrode system and a hydrophilic high molecular substance layer or a layer composed of a hydrophilic high molecular substance and an inactivated oxidoreductase being provided on another electrode system.
10. A biosensor as claimed in claim 2 or 3, wherein said electrode system comprises two sets of electrode systems comprising at least an electrode for measurement and a counter electrode mainly composed of carbon, a reaction layer composed of a hydrophilic high molecular substance and an oxidoreductase being provided on one electrode system and a hydrophilic high molecular substance layer or a layer composed of a hydrophilic high molecular substance and a deactivated oxidoreductase being provided on another electrode system.
11. A biosensor as claimed in claim 9 or 10, wherein said electrode system comprises an electrode for measurement and a counter electrode mainly composed of carbon and a reference electrode comprising a silver/silver chloride reference electrode.

12. A biosensor as claimed in claim 1, wherein said electrode system is prepared from a material mainly composed of carbon formed on an insulating base plate by means of screen printing.

13. A biosensor as claimed in claim 1, wherein an electrode system comprising at least an electrode for measurement and a counter electrode is formed on an insulating base plate, a reaction layer is formed on said electrode system and said reaction layer comprises a first layer composed of a hydrophilic high molecular substance and an oxidoreductase, a second layer composed of a hydrophilic high molecular substance and a third layer containing an electron acceptor.

14. A biosensor as claimed in claim 13, wherein said hydrophilic high molecular substance in the first layer and the second layer is selected from hydrophilic high molecular substances of starch, carboxymethyl cellulose, gelatin, acrylate, vinyl alcohol, vinylpyrrolidone and maleic anhydride or a mixture thereof.

15. A process for preparing a biosensor which comprises forming an electrode system comprising at least an electrode for measurement and a counter electrode on an insulating base plate, coating a hydrophilic high molecular substance aqueous solution and an oxidoreductase aqueous solution on said electrode system and then drying to form an enzyme layer, spreading a mixture of an electron acceptor and an organic solvent onto said enzyme layer,

removing said organic solvent to form an electron acceptor layer and then integrating together with a cover.

16. A process for preparing a biosensor which comprises forming an electrode system comprising at least an electrode for measurement and a counter electrode on an insulating base plate, coating a hydrophilic high molecular substance aqueous solution and an oxidoreductase aqueous solution on said electrode system and then drying to form an enzyme layer, next spreading a solution of a hydrophilic high molecular substance in an organic solvent onto said enzyme layer to form a hydrophilic high molecular substance layer, further spreading a dispersion of an electron acceptor in an organic solvent onto said hydrophilic high molecular substance layer to form an electron acceptor layer, and then integrating together with a cover.

17. A process for preparing a biosensor as claimed in claim 15 or 16, wherein a mixture of said electron acceptor, said surface active agent and said organic solvent is spread on said enzyme layer and said organic solvent is removed to form an electron acceptor layer.

18. A process for preparing a biosensor as claimed in claim 17, wherein said electron acceptor layer comprises fine particles of an electron acceptor having a particle size of not greater than 100  $\mu\text{m}$ .

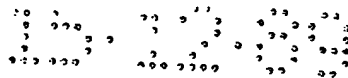


FIG. 1

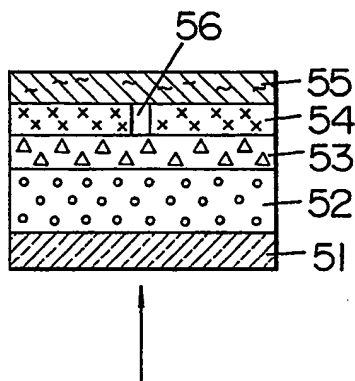
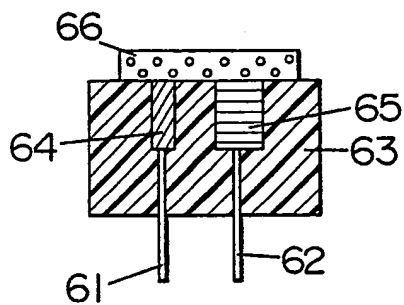


FIG. 2



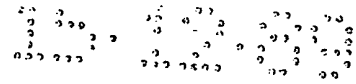
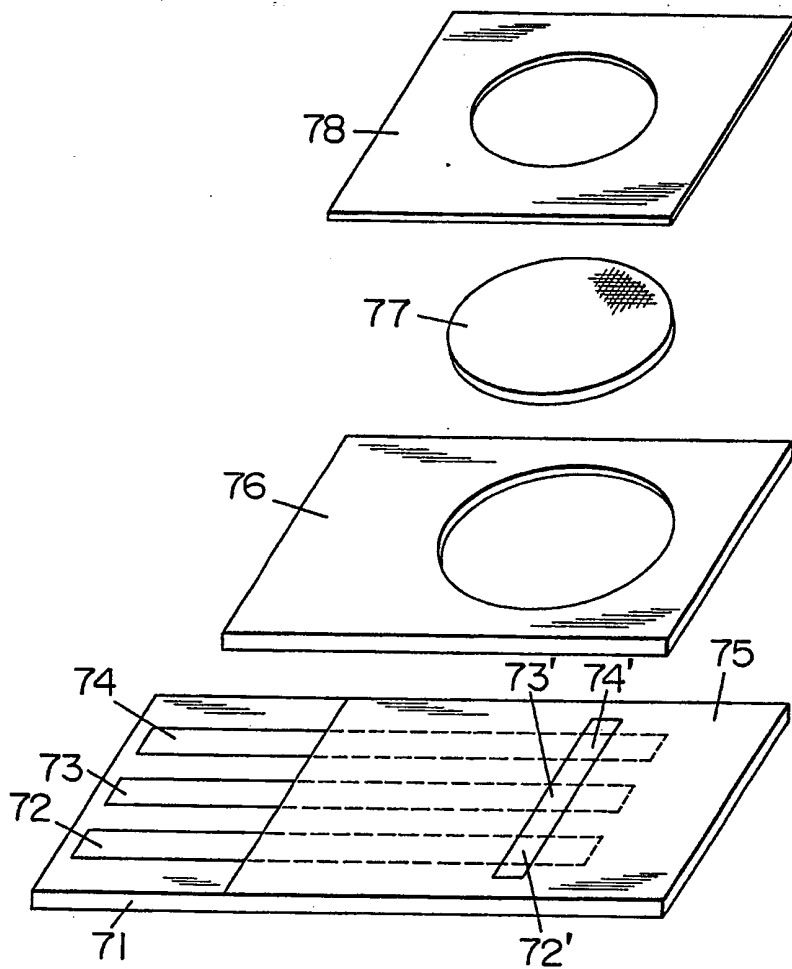


FIG. 3





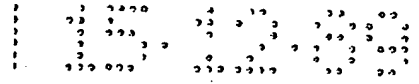
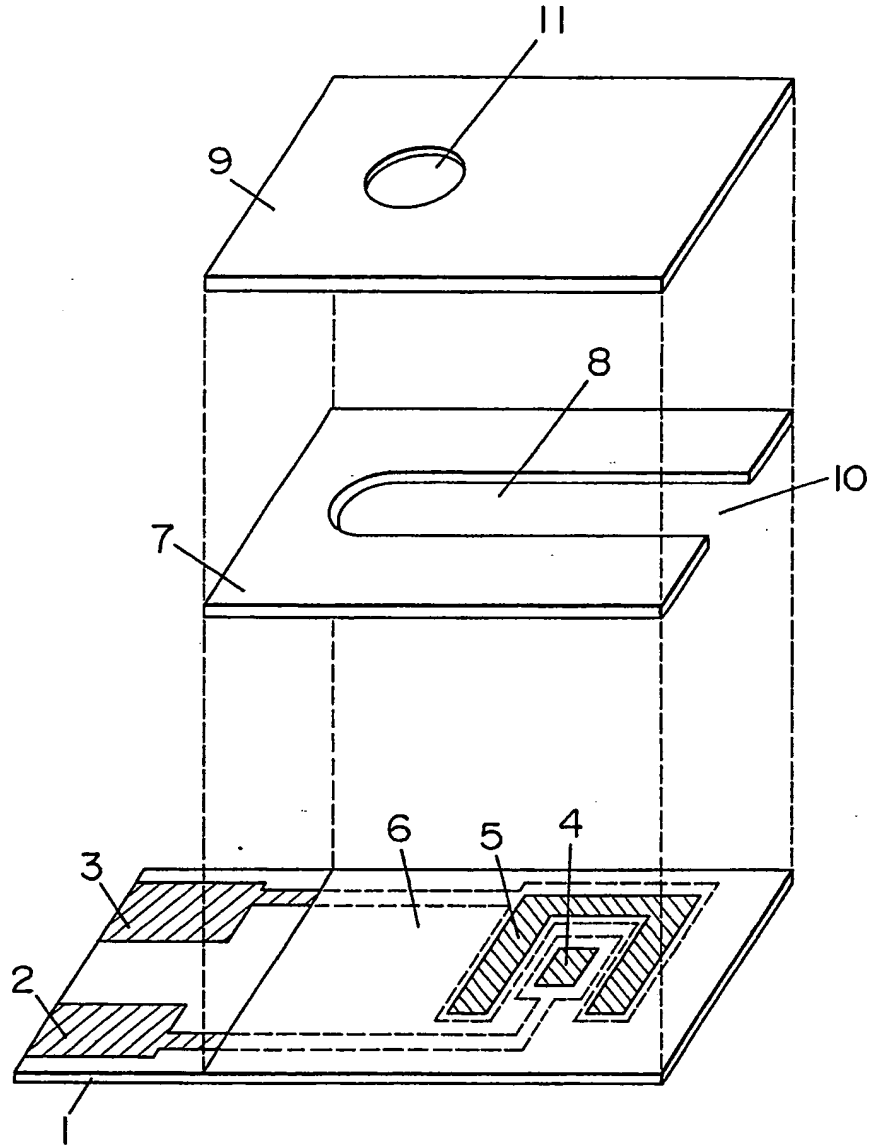


FIG. 4



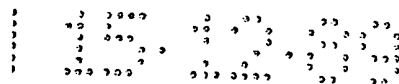


FIG. 5

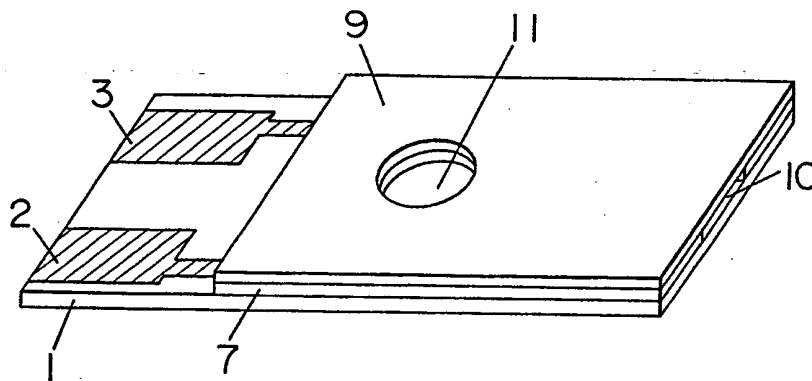
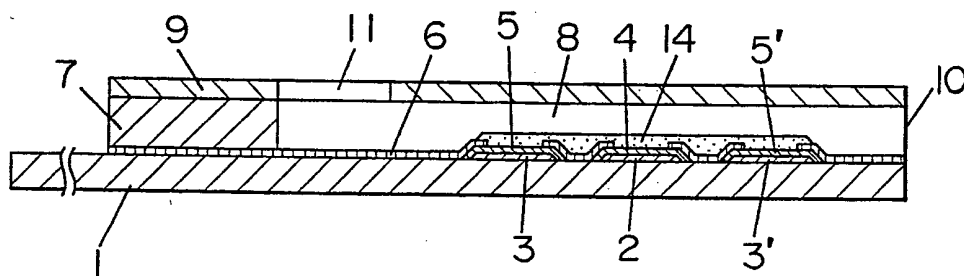


FIG. 6



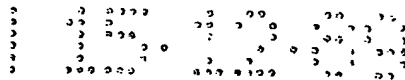
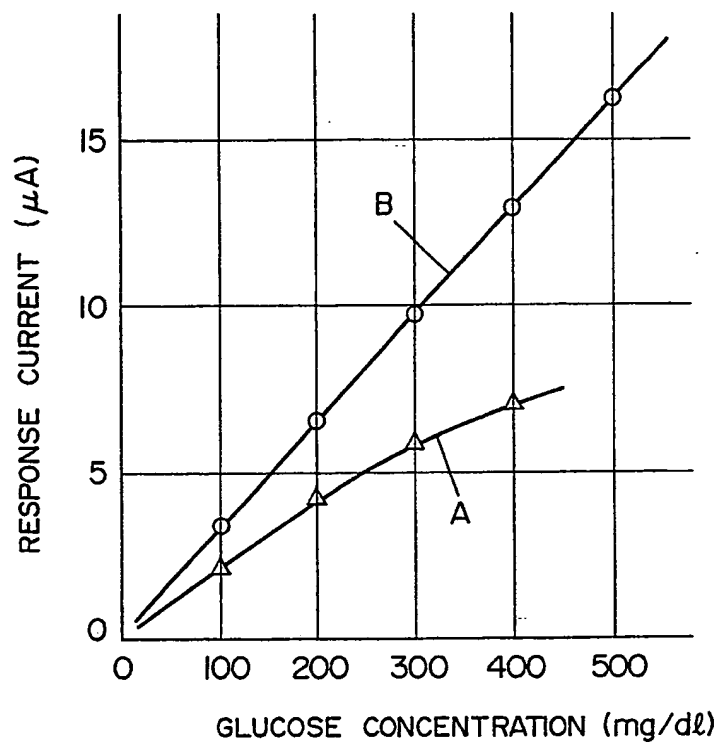


FIG. 7



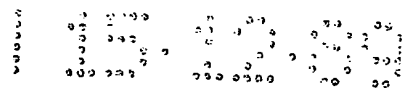
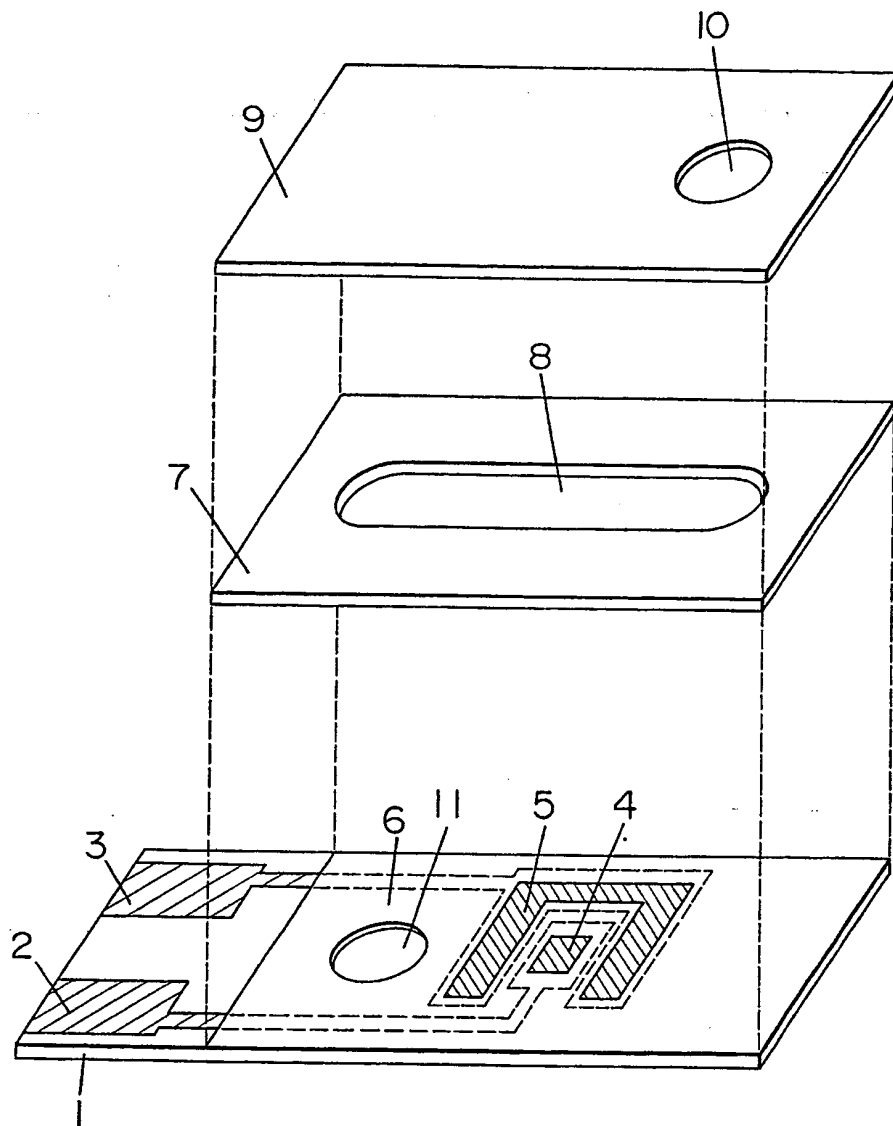


FIG. 8



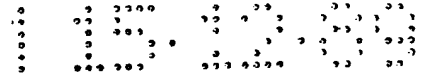
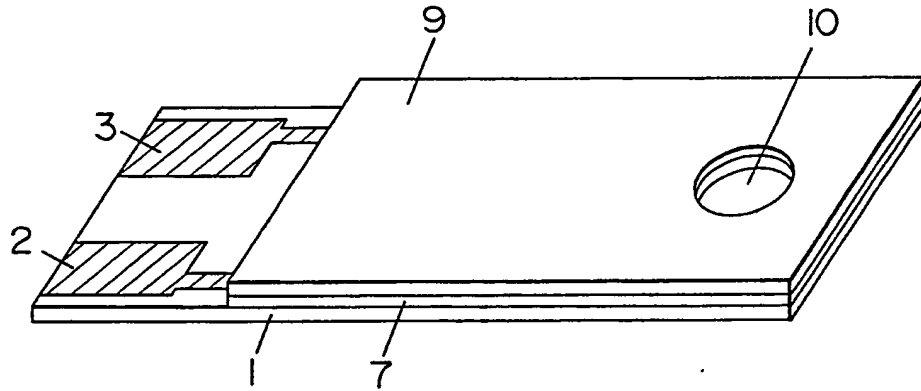


FIG. 9



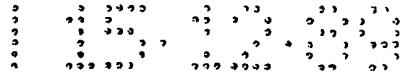
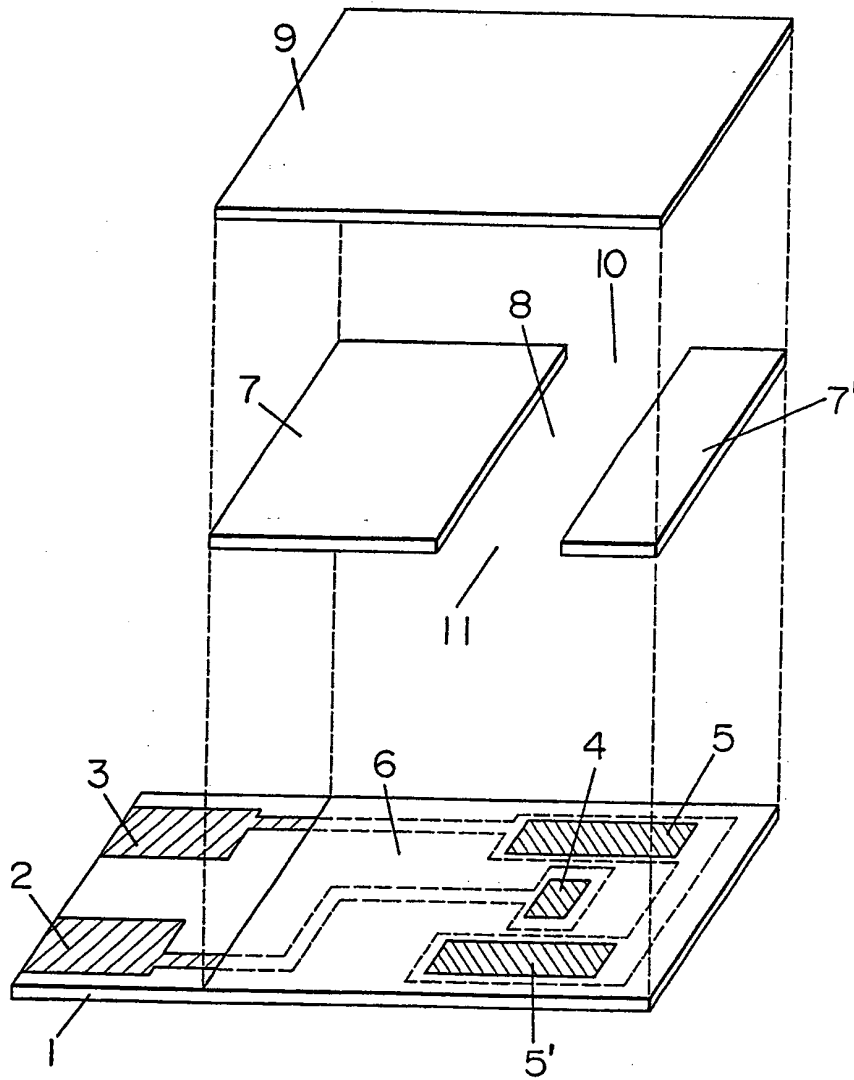


FIG. 10



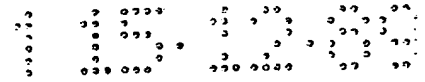
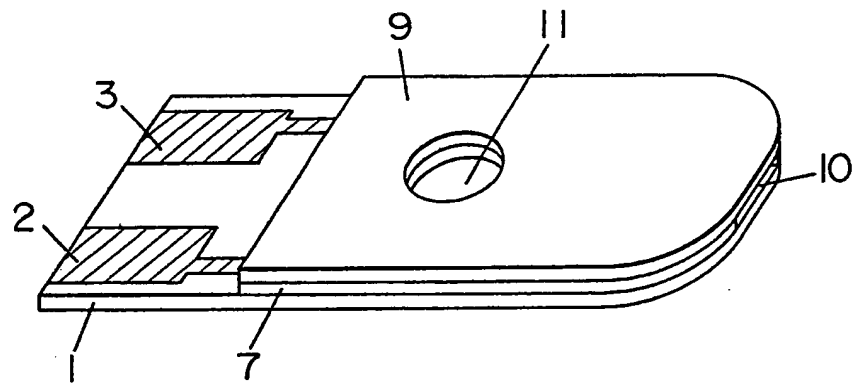


FIG. II



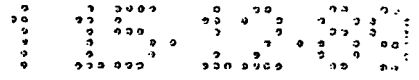
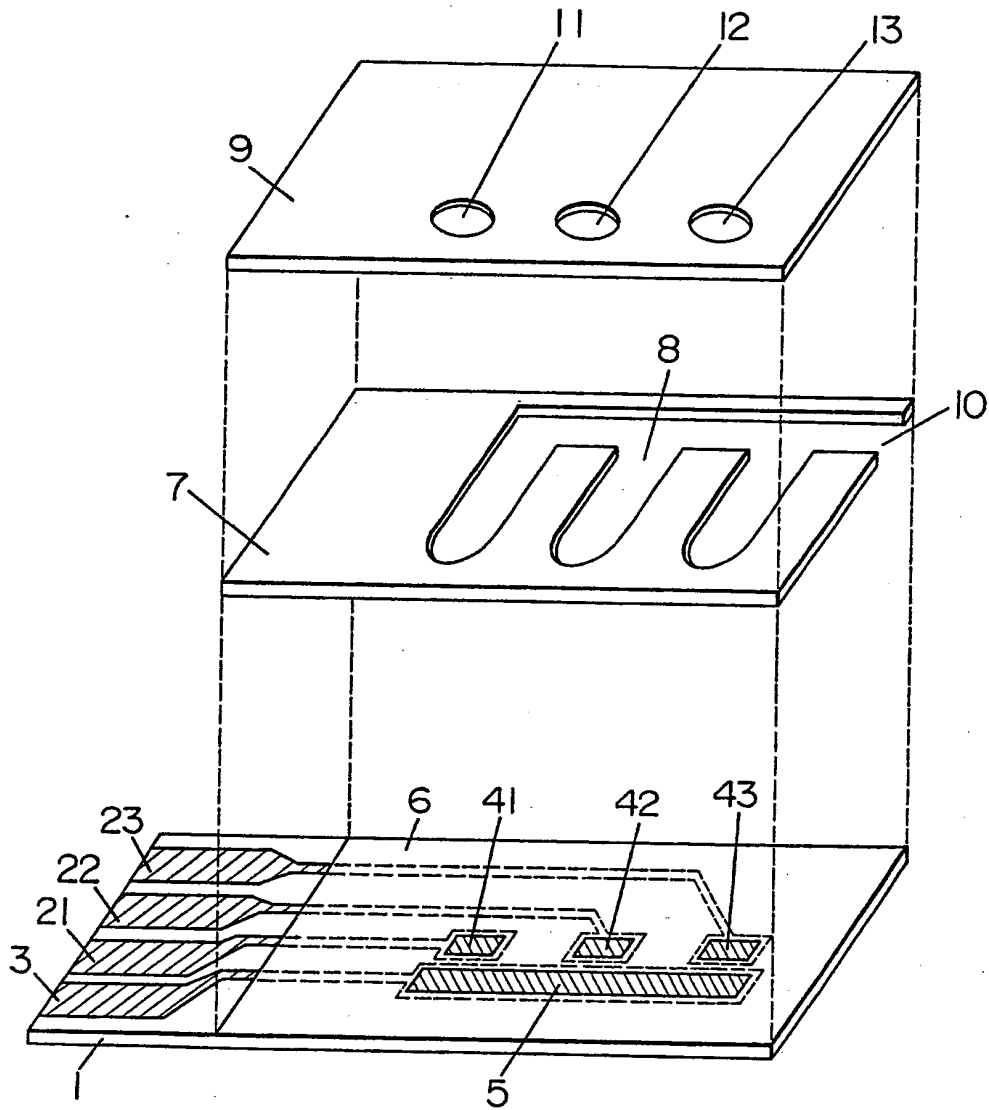


FIG. 12





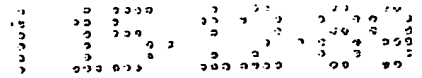
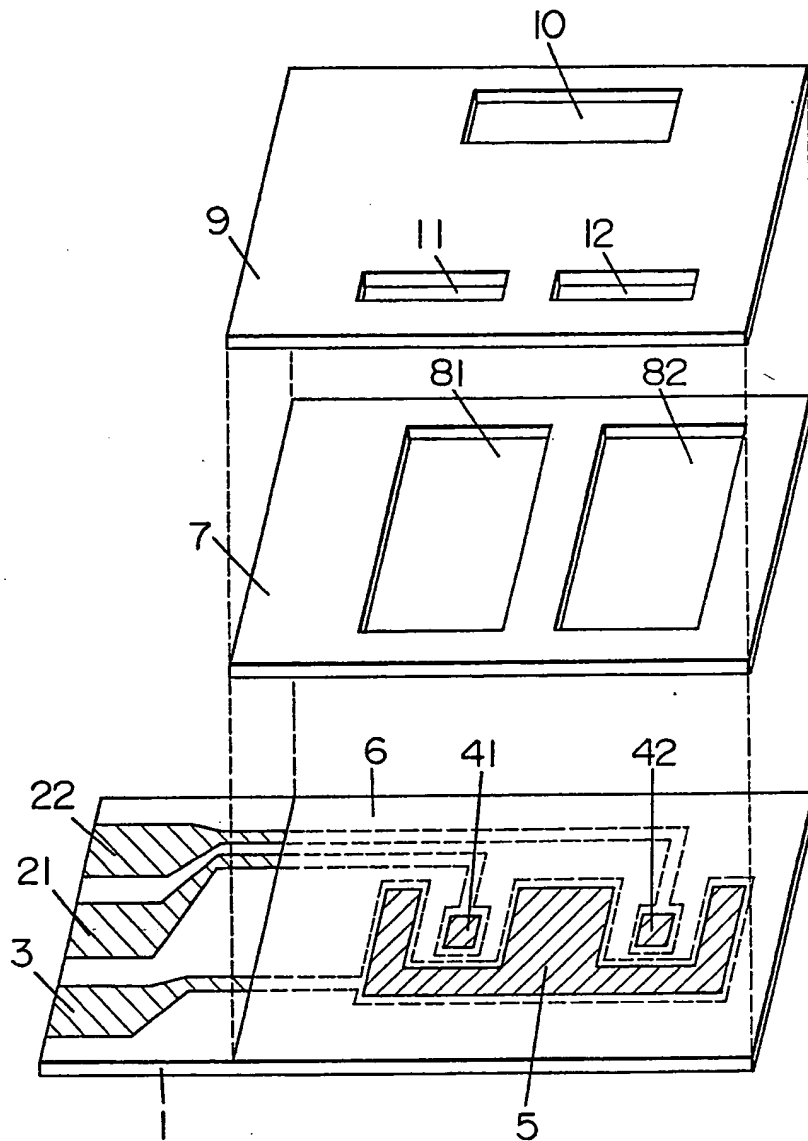


FIG. 13



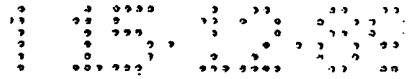
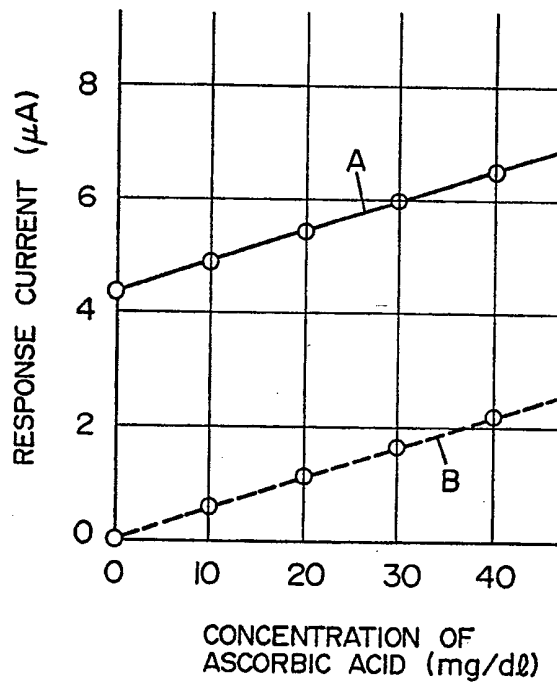


FIG. 14



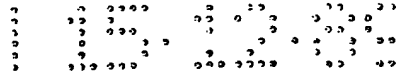
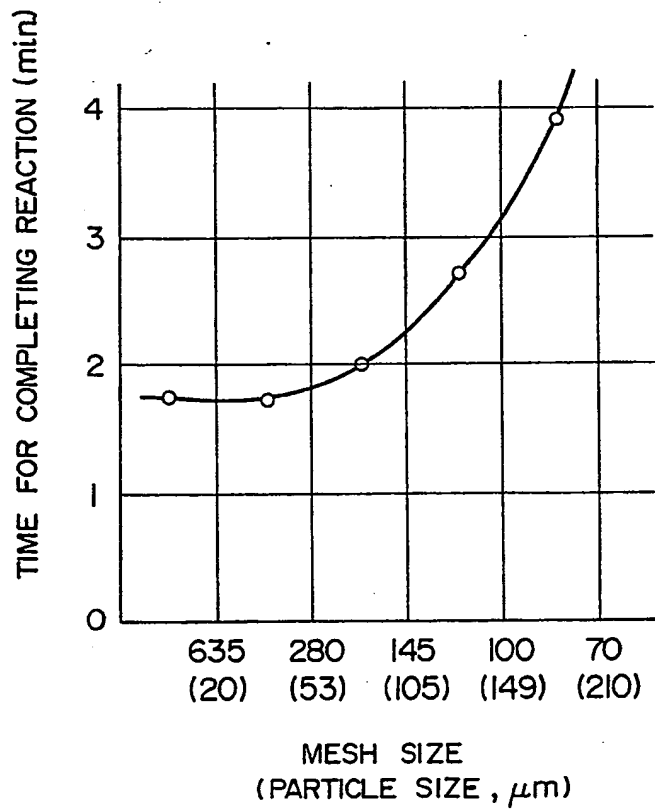
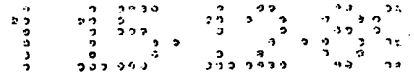


FIG. 15





List of numerals appearing in the drawings:

- 1, 63, 71 ..... base plate
- 2, 3, 3', 21, 22, 23, 61, 62, 72, 73, 74 ... lead
- 4, 41, 42, 43, 64, 73 ..... electrode for measurement
- 5, 5', 65, 72 ..... counter electrode
- 6, 75 ..... insulating layer
- 7, 7' ..... spacer
- 8, 81, 82 ..... space
- 9, 78 ..... cover
- 10 ..... introducing port
- 11, 12, 13 ..... discharge port
- 14 ..... reaction layer
- 51 ..... support
- 52 ..... reagent layer
- 53 ..... spreading layer
- 54 ..... waterproofing layer
- 55 ..... filtering layer
- 55 ..... pore
- 66, 77 ..... porous material
- 74 ..... reference electrode
- 76 ..... holding frame

# INTERNATIONAL SEARCH REPORT

International Application No PCT/JP89/00337

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl <sup>4</sup> G01N27/28, G01N27/30		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC	G01N27/28-27/30, 27/46	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
Jitusyo Shinan Koho                      1926 - 1989		
Kokai Jitsuyo Shinan Koho            1971 - 1989		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	JP, A, 62-137559 (Matsushita Electric Ind. Co., Ltd.) 20 June 1987 (20. 06. 87) (Family: none)	1-18
A	JP, A, 63-3248 (Matsushita Electric Ind. Co., Ltd.) 8 January 1988 (08. 01. 88) (Family: none)	1-18
A	JP, A, 63-58149 (Matsushita Electric Ind. Co., Ltd.) 12 March 1988 (12. 03. 88) (Family: none)	1-18
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
June 26, 1989 (26. 06. 89)		July 10, 1989 (10. 07. 89)
International Searching Authority		Signature of Authorized Officer
Japanese Patent Office		

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